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The Rf and Rf-like PPR in higher plants, a fast-evolving subclass of PPR genes

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In the last years, a number of nuclear genes restoring cytoplasmic male sterility (CMS) have been cloned in various crop species. The majority of these genes have been shown to encode pentatricopeptide repeat proteins (PPR) that act by specifically suppressing the expression of sterility-causing mitochondrial transcripts. Functional analysis of these proteins has indicated that the inhibitory effects of restoring PPR (Rf-PPR) proteins involve various mechanisms, including RNA cleavage, RNA destabilization, or translation inhibition. Cross-species sequence comparison of PPR protein complements revealed that most plant genomes encode 10–30 Rf-like (RFL) proteins sharing high-sequence similarity with the identified Rf-PPRs from crops. Evolutionary analyses further showed that they constitute a monophyletic group apart in the *PPR* family, with peculiar evolution dynamic and constraints. Here we review recent data on RF-PPRs and present the latest discoveries on the RFL family, with prospects on the functionality and evolution of this peculiar subclass of PPR.

Cytoplasmic Male Sterility, A Valuable Genetic Model to Study Nucleo-Mitochondrial Interactions in Higher Plants

Besides being involved in essential functions like respiration or cofactor biosynthesis, plant mitochondria are also responsible for a trait called cytoplasmic male sterility (CMS). This trait corresponds to a widely distributed condition of plants manifested by an inability to produce functional pollen.[1] In nature, CMS can be observed in gynodioecious populations of wild species in which both hermaphrodites and male-sterile (female) individuals co-habit. CMS can also appear in progenies of inter-specific crosses in which the nuclear background of one species is combined with cytoplasmic background of another. In these cases, the male sterile phenotype can either result from disruption of cyto-nuclear co-adaptation and deregulation of multiple genetic factors or from the reactivation of a single mitochondrial gene that was silenced when combined with its original nucleus. The first type of CMS is thought to result from a loss of function mechanism whereas the second situation is interpreted more like a gain of function associated with the revival of a dormant CMS (for examples, see ref. 2). Since the male-sterile condition forces plant out-crossing, CMS have been transferred from wild species into a number of cultivated plant species and extensively used by the seed industry to reduce the costs associated with hybrid seed production. The strong agronomical interest for CMS has allowed the development of extensive research programs aiming at deciphering the physiology of this trait as well as identifying the involved genetic determinants. In the last years, important progress has been made to understand the nuclear control of CMS and several nuclear loci regulating the expression of mitochondrial CMS determinants have been identified in various crop species.

Gain-of-function CMS are always associated with the expression of poorly conserved and often chimeric open reading frames (ORFs) found on mitochondrial genomes. These ORFs are generally co-transcribed with essential mitochondrial genes, and this association likely favors the stability and the maintenance of CMS genes within mitochondrial genomes.[3] The way CMS-inducing proteins interfere with pollen development and why their effects inhibit only the production of the male gametophyte despite being constitutively expressed are still largely unclear. The cytotoxicity of several CMS-inducing proteins when expressed in *E. coli* strongly suggests that these proteins may be detrimental to mitochondrial respiration.[4-6] A few CMS-causing proteins were effectively shown to moderately impair respiratory chain activity or to form large pores within the mitochondrial inner membrane, but how this correlates with pollen abortion remains elusive.[7-11]

Male fertility can be restored by the expression of nuclear-encoded genes called restorer of fertility (*Rf*), which re-establish partial to normal pollen production to plants carrying a corresponding sterility-inducing cytoplasm. Fertility restoration is generally associated with a strong reduction in the production of mitochondrial CMS-inducing proteins. Perturbations in the abundance or in the pattern of the CMS-associated transcripts are also often observed. However, the exact molecular mechanisms resulting in the loss of the CMS-inducing protein have not been resolved in most cases. In the last years, several *Rf* genes were cloned from various crop species and the majority of them encode proteins belonging to the pentatricopeptide repeat family (PPR) (summarized in **Table 1**).[6,12-17] All these restoring PPR proteins (hereafter called Rf-PPR) were shown to target the mitochondrion and act by specifically reducing the accumulation of their cognate CMS-associated mitochondrial RNAs and/or proteins. The prevalence of PPR proteins in plastid and mitochondrial RNA processing is now largely documented[18] and the fact that many *Rf* genes were found to encode PPR proteins is coherent

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Table 1. Functionally characterized PPR-protein-encoding restorer of fertility genes (*Rf-PPR*) and highly related *RFL* genes that do not support restoration activity

Species	CMS	*Rf* or *Rf-like* gene	Type of PPR	# of aa	# of repeats	Mitochondrial target	Associated molecular mechanims	Refs.
Cloned *Rf-PPR* genes								
Petunia hybrida sp.		*Rf-PPR592*	P	592	14	*urfS*	RNA processing or translation inhibition?	Bentolila 2002
Raphanus sativus	Ogura	*Rfo (=Rfk1 or PPR-B)*	P	687	17	*orf138*	translation inhibition	Brown et al., 2003; Desloire et al. 2003
Raphanus sativus	Kosena	*Rfk1 (=Rfo or PPR-B)*	P	687	17	*orf125*	translation inhibition	Koizuka et al., 2003
Oryza sativa	BorolI	*Rf1a*	P	791	18	*orf79*	RNA processing	Kazama et al., 2003; Komori et al., 2003; Akagi et al., 2004; Wang et al., 2006
Oryza sativa	BorolI	*Rf1b*	P	506	11	*orf79*	RNA degradation	Wang et al., 2006
Oryza sativa	Honglian	*Rf5=Rf1a*	P	791	18	*orfH79*	RNA processing	Hu et al., 2012
Sorghum bicolor	A1	*Rf1*	PLS	628	14	?	RNA editing?	Klein et al., 2005
***Rf* genes suspected to encode a PPR protein**								
Mimulus guttatus	IM62	*Rf1*	P?	?	?	*orf141?*	RNA processing?	Barr et al., 2010
Mimulus guttatus	IM62	*Rf2*	P?	?	?	*orf141?*	RNA processing?	Barr et al., 2010
Sorghum bicolor	A1	*Rf2*	P?	?	?	?	?	Jordan et al., 2010
Sorghum bicolor	A2	*Rf5*	P?	?	?	?	?	Jordan et al., 2011
Functionally characterized *RFL* genes								
Arabidopsis thaliana	NA	*RPF1*	P	602	14	*nad4*	RNA processing	Hölzle et al., 2011
Arabidopsis thaliana	NA	*RPF2*	P	630	16	*nad9, cox3*	RNA processing	Jonietz et al., 2010
Arabidopsis thaliana	NA	*RPF3*	P	629	15	*ccmC*	RNA processing	Jonietz et al., 2011
Arabidopsis thaliana	NA	*NG1*	P	485	12	?	?	Yang et al., 2011

Rf genes mapping within clusters of *RFL* genes and that are therefore strongly suspected to encode a PPR protein are also indicated. NA, not applicable.

with the roles these proteins exert on CMS-inducing mitochondrial mRNAs. PPR proteins constitute a large family of RNA binding proteins characterized by the succession of degenerate motifs of approximately 35 amino-acids.[19,20] PPR tracks organize highly specific interaction domains, which preferentially associate with single-stranded RNAs.[21] Functional characterization of Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), rice (*Oryza sativa*), and *Physcomitrella patens* mutants has revealed the plethoric roles played by PPR proteins in organellar gene expression. These proteins were shown to participate in most RNA processing and expression steps including gene transcription, RNA stabilization, 5' and 3' RNA cleavage, intron splicing, RNA editing, and mRNA translation.[18] Except maybe for those involved in RNA editing, PPR protein activity seems limited to an ability to specifically associate with short RNA segments. It was recently shown that PPR binding could induce local RNA structure reorganization to make otherwise hidden binding sites more accessible to other proteins or complexes.[22] The ability of PPR proteins to locally influence RNA secondary or tertiary structures likely explains the large variety of RNA processing steps associated with this class of proteins.

Unlike most mitochondria-associated functions in obligate aerobes, the non-essentiality of the CMS phenotype has allowed CMS/Rf systems to serve as precious models to study nucleo-mitochondrial genetic interactions in plants. Over the last years, functional characterization of Rf-PPR proteins has greatly participated in the comprehension of PPR protein molecular activity and demonstrated that Rf-PPR proteins interfere with the expression of CMS-causing mRNAs by driving the same RNA processing mechanisms as for positively-acting PPR proteins. Sequence alignments have also revealed that most identified Rf-PPRs show strong sequence similarity with a group of 10–30 PPR proteins encoded in the species in which they were found or in closely related species.[13,23] These proteins constitute the subgroup of Rf-like (RFL) PPR proteins. Recent large-scale phylogenetic analyses have further shown that RFL and Rf-PPR proteins constitute an evolutionary distinct subgroup of proteins within the PPR family in angiosperms and that all identified *Rf-PPR* genes have evolved from this unique group of *PPR* genes.[24,25]

Rf-PPR Genes Impede Specifically the Expression or the Accumulation of CMS-Associated Transcripts

The cloning of the *Rf-PPR592* gene from petunia was the first one revealing that *Rf* genes can encode mitochondria-targeted PPR proteins.[12] In petunia, a single cytoplasm is known to

induce CMS and the *pcf* mitochondrial gene was shown to be responsible for the expression of the CMS trait in this system.[26] *pcf* encodes a 43-kDa chimeric protein comprising portions of the ATP synthase subunit 9 and the cytochrome oxidase subunit 2 fused to an ORF of unidentified origin named *urfS*. Several transcripts bearing different 5' ends are derived from the *pcf* locus, and the ones with 5' termini mapping 121 nucleotides upstream of the AUG codon exhibit strong reduction in restored plants.[27] Genetic analyses have indicated that a single dominant nuclear gene, *Rf*, restores male fertility to petunia CMS plants.[28] The *Rf* locus was cloned by positional cloning and revealed to contain two highly homologous *PPR* genes that were named *Rf-PPR592* and *Rf-PPR591*.[12] These two genes result from a recent duplication and encode proteins comprising 14 PPR repeats that are 93% similar. Both Rf-PPR592 and Rf-PPR591 proteins can be transported to mitochondria, but only Rf-PPR592 is able to restore male fertility to petunia CMS plants. Transgenic analysis confirmed the ability of Rf-PPR592 to restore pollen production by decreasing the amount of the PCF protein. A tagged version of Rf-PPR592 was shown to co-sediment with the *pcf* transcript in large multi-protein complexes and co-immunoprecipitation experiments supported an interaction between Rf-PPR592 and *pcf* transcript in vivo.[29] A region of *pcf* 5' leader located between 101–342 nucleotides upstream of the AUG codon showed greatest enrichment in the immunoprecipitated material, indicating that Rf-PPR592-binding site might be located in this region of *pcf* transcript. These results did not allow to firmly conclude on Rf-PPR592 mode of action yet but the association with the *pcf* mRNA 5'UTR supports a potential role in *pcf* transcript 5'-end processing as previously suspected.[27] It also remains possible that this processing prevents proper translation of *pcf* mRNA, implying an indirect role of Rf-PPR592 in PCF translation.

Soon after the identification of the petunia restorer, the *Rfo* and *Rfk1* genes restoring fertility to the Ogura and the Kosena radish (*Raphanus sativus*) cytoplasms, respectively, were simultaneously identified by three different groups.[13-15] These sister CMS are caused by the *orf138* (Ogura) or the *orf125* (Kosena) mitochondrial genes, which encode almost identical sterility-inducing proteins showing no homology with any other known proteins.[30-32] Both CMS originates from radish and were successfully transferred to rapeseed (*Brassica napus*) by protoplast fusion. Cloning of the associated restorer genes showed that Ogura and Kosena CMS *Rf* loci are identical and encode three highly similar PPR proteins out of which only one, PPR-B, carries the restoration activity.[13-15] *PPR-B* encodes a protein composed of 687 amino acid and 17 PPR repeats that is partly associated with mitochondrial membranes.[33] Expression analysis in rapeseed transgenic plants showed that PPR-B protein accumulates preferentially in anthers of restored plants. Moreover, immunolocalization of ORF138 revealed that the complete removal of ORF138 from the tapetum and the microspores is critical for fertility restoration and indicates that PPR-B principal sites of action reside in these tissues.[33] Molecular events associated with fertility restoration do not impact the size or the abundance of the *orf138* mRNA, even in a tissue-specific manner.[31,33,34] Additional analyses showed that the *orf138* mRNA co-immunoprecipitates specifically with the PPR-B protein.[33] These data favor a post-transcriptional role of PPR-B in *orf138* mRNA expression and the current view on PPR-B functioning is that it may impede *orf138* mRNA translation by preventing either association or progression of mitochondrial ribosomes with the *orf138* mRNA.[33]

In rice, nuclear restorers controlling the expression of two genetically independent CMS systems were also recently identified. Two of the recognized restorer genes re-establish male fertility to the Boro II-type (BT) CMS.[35] In this system, the male sterility is induced by the B-*atp6* mitochondrial locus, which comprises a copy of the *atp6* gene and an additional downstream sequence containing a predicted ORF called *orf79*.[36,37] The 5' region of *orf79* is similar to the rice mitochondrial *cox1* gene, whereas the rest of the gene shows no homology with any known sequence. Fertility restoration of the BT-type CMS is associated with the *Rf-1* dominant locus,[35,38] independently cloned by several research groups. Although it was named differently, they all identified the same gene, ultimately called *Rf1a*, encoding a PPR protein of 791 amino acids and constituted of 18 PPR repeats.[6,39-41] *Rf1a* was initially considered to be the sole gene of the *Rf-1* locus restoring the BT-type CMS, but further exploration of the *Rf-1* genomic region revealed a second *PPR* gene also functioning in fertility restoration.[6] This second restorer was named *Rf1b* and is likely not present in all rice restorer lines. *Rf1b* encodes a protein of 506 amino acids forming 11 PPR repeats and shares 70% of identity with RF1A. Both RF1A and RF1B proteins are targeted to rice mitochondria, but they block the production of the ORF79 cytotoxic protein by two distinct mechanisms. RF1A directs endonucleolytic cleavages at three major regions within the B-*atp6*/*orf79* mRNA liberating the B-*atp6* and *orf79* ORFs in the form of monocistronic transcripts.[6] Additional experiments indicated that the most abundant cleavage product associated with RF1A bears a 5' end located 52 nucleotides upstream from the translation initiation codon of the *orf79* transcript[36,42,43] and that RF1A binds to a short RNA segment preceding this processing site with high affinity in vitro.[43] Moreover, liberated monocistronic *orf79* transcripts showed no association with mitochondrial polysomes, strongly supporting that they are not translated in vivo. Taken together, these results suggest that the primary function of RF1A is to induce an endonucleolytic cleavage upstream of the *orf79* ORF and that this processing impacts negatively the translation status of *orf79* transcript. A slight destabilization of the processed *orf79* transcript in the presence of *Rf1a* may participate in successful maturation of pollen, but it could be simply consecutive of the inability to translate the *orf79* monocistronic mRNA. Oppositely to *Rf1a*, *Rf1b* mediates complete degradation of the B-*atp6*/*orf79* dicistronic mRNA and the way this degradation is orchestrated is presently unclear.[6] The second and normal copy of *atp6* is not affected by *Rf1b* suggesting that the post-transcriptional destabilization of B-*atp6*/*orf79* may proceed through sequences present within the *orf79* ORF. Surprisingly, *Rf1a* was found to be epistatic over *Rf1b*. In the presence of both restorer genes, the B-*atp6*/*orf79* dicistronic mRNA is effectively preferentially cleaved by RF1A and the generated cleavage products are not further destabilized by the action of RF1B.

The rice *Rf5* gene, restoring the Honglian (HL) CMS, has also been recently recognized and analyzed at the molecular level.[17,44] The identified gene encodes a PPR protein identical to RF1A, but surprisingly the mechanism associated with HL CMS restoration differs from the BT-type CMS. Although the HL-CMS is caused by a mitochondrial gene that is almost identical to *orf79*[45] and that fertility restoration in HL-CMS also involves an endoribonucleolytic cleavage upstream of *orf79H,* RF5 (RF1A) does not have the ability to bind to the *atp6-orfH79* transcript intergenic region in vitro.[17] It has been proposed that nucleotide variations between *atp6-orf79* and *atp6-orfH79* transcripts may be responsible for the inability of RF5 to bind upstream of *orfH79*. The search for RF5 protein partners identified the glycine rich 162 protein (GRP162) and further analysis indicated that this protein plays essential role in the processing of the *atp6-orfH79* bi-cistronic transcript. GRP162 contains two RNA recognition motifs (RRM) and was shown to associate specifically with two different regions within the *atp6-orfH79* intergenic region. Interestingly, GRP162 and Rf5 were shown to be components of a fertility restoration complex of about 500 kDa. Whether these two proteins contribute to establish the RNA-binding specificity of this complex and help in the recruitment of a possible endoribonuclease responsible for *atp6-orfH79* mRNA cleavage remains to be determined. This analysis is interesting as it revealed that a same restoring PPR protein can silence near-identical CMS genes through the same molecular mechanism but involving different protein partners. It also documents that Rf-PPRs can act in multi-protein complexes, and that the RNA-binding specificity of restoration complexes may be not attributable only to the involved Rf-PPR proteins. Interestingly, co-operation of PPR and GRP proteins in establishing restoration activity may concern other CMS systems as one restorer gene of the lead rice-type CMS was recently shown to encode a mitochondria-targeted glycine rich protein.[46]

In sorghum, the *Rf1* gene which restores CMS to the A1 cytoplasm was also tentatively cloned by high-resolution mapping.[16,47] The *Rf1* locus was delimited to a 32-kb region containing four ORFs among which the *PPR13* gene represents the best gene candidate supporting fertility restoration activity. Opposite to all known *Rf-PPR* genes, *PPR13* encodes a PPR protein belonging to the PLS subgroup and contains 14 PPR repeats as well as a C-terminal E motif.[20] In addition to the canonical 35-amino-acid P repeats, PLS-type PPR proteins contain shorter (S) and longer (L) PPR repeats as well as conserved C-terminal domains. These type of PPR have been almost exclusively associated to C-to-U RNA editing in both plastids and mitochondria.[18] Additional analyses are required to confirm that PPR13 corresponds effectively to *Rf1*, but if this is verified this would indicate that the fertility restoration in the A1 cytoplasm involves the editing of a mitochondrial transcript. Given that the mitochondrial gene inducing the A1 CMS has not been discovered yet, the implication of RNA editing in the restoration process of this CMS cannot be confirmed for the moment. It should also be emphasized that *PPR13* shares no significant homology with the petunia, rice, and radish restorers (see above) and, thus, cannot be assimilated as an *RFL* gene.

Finally, a number of as-yet-unidentified restorer genes are strongly suspected to encode PPR proteins since preliminary analyses indicate that they map to chromosomal regions containing one or several *Rf-like PPR* genes. This includes a second restorer gene (*Rf2*) of the sorghum A1 CMS, which was resolved to a 236 kb region.[48] One of the genes in the identified interval encodes a protein highly homologous to the rice *Rf1a* gene (see above) and represents a strong potential candidate for *Rf2*. A similar situation was encountered for *Rf5* restoring the A2 CMS in sorghum.[49] This locus was mapped to a 584 kb DNA segment and a cluster of six *PPR* genes exhibiting strong homology with the rice *Rf1a* gene was also identified. Previous genetic analyses showed that the *Rf5* gene was able to restore both the A1 and A2 CMS, and it was postulated that multiple *PPR* within this locus might correspond to active restorer genes. Similarly, the two genetically linked *Rf1* and *Rf2* genes mapped in *Mimulus guttatus* were found to reside in chromosomal loci containing 12 and six *Rf-like* genes, respectively.[50] As previously reported for the BT-type CMS in rice (see above), two different dominant *Rf* alleles appear to cohabit in the analyzed *Mimulus guttatus* line. This observation further supports the concept that multiple genetically linked *Rf* genes restoring a same CMS can be found at some restorer loci. In all these cases, further analyses, including functional study of the encoded proteins, are necessary to identify which of these *Rf*-like genes carry the restoration activity.

Characterized Arabidopsis Rf-like PPRs are Involved in Mitochondrial mRNA 5' end Processing

Several studies on the *PPR* complement in various terrestrial plant genomes pointed out a small subgroup of proteins that share high similarity with the identified Rf-PPRs, rendering them more homologs to each other across plant lineages than to other PPR proteins.[13,23,24] On the basis of these homologies, *RFL* genes could be identified in a number of angiosperm species.[25] Compared with the large size of the PPR family in terrestrial plants,[18,51] they constitute a relatively small subgroup of genes, ranging from five predicted *RFL* genes in *Zea mays* to 33 in *Arabidopsis lyrata*.

Several characteristics make the *RFL* subfamily stand apart from the bulk of plant *PPR* genes. Unlike most *PPR* genes, for which interspecies orthologous relationships can be easily found, *RFL* genes form species-specific paralogous groups.[23] The conservation of *PPR* genes throughout evolution, with limited loss or gain, strongly suggests that they are under conservative selection pressure, which is indicative of their essential roles in organellar functioning, predating the divergence of monocots and dicots.[23] On the contrary, the apparent non-conservation of *RFL* genes underlines diversifying constraints.[25] Another striking difference between *PPR* and *RFL* genes concerns their genomic organization. Whereas *PPR* genes are randomly dispersed throughout plant genomes, *RFL* genes seems to be mainly clustered in non-conserved genomic locations. In *Arabidopsis thaliana*, most *RFL* genes are mainly clustered in two regions of chromosome 1, and they are mostly found on rice chromosome 10.[20,23,51] This is consistent with the observations showing that *Rf* genes generally map to *PPR* gene-rich regions in several CMS systems.[6,13,14,48-50] The

physical clustering of highly homologous *RFL* genes is indicative of extensive and ongoing local duplication of genes belonging to this subfamily. The *RFL* subfamily likely represents a reservoir of actively evolving *PPR* genes through a "birth-and-death" selection process implying dynamic local gene duplications, interallelic recombination, gene conversion, and ultimately selection for functional divergence. This contrasts with the rest of the *PPR* family, which likely expanded mostly through retrotransposition.[23] Moreover, the diversifying selection has probably been directed toward specific residues in the RFL-embedded PPR motifs, leading to a modification of RNA-binding specificities, hence expanding the possibilities of such binding.[25] Lastly, this subfamily obviously originated from a unique common ancestor, predating the separation of monocots and dicots, since *RFL* genes have been found in a large variety of angiosperms species.

To date, only three RFL proteins not related to CMS suppression have been functionally characterized in *Arabidopsis thaliana* (**Table 1**). Their identification resulted from a comparative analysis of major 5' and 3' termini of mitochondrial transcripts in different *Arabidopsis thaliana* accessions.[52] This study revealed that mitochondrial mRNAs are differentially processed at their 5' ends between Arabidopsis accessions. Genetic analysis further indicated that most of these differences were governed by single dominant nuclear loci. The first characterized *RFL* gene corresponded to the *RNA Processing Factor 2* (*RPF2*) and was shown to be responsible for the production of a *nad9* mRNA 5' end located 202 nucleotides upstream of the translation initiation codon in Arabidopsis Columbia (Col.0) and Landsberg erecta (Ler) accessions.[53] Knockout mutant analysis further revealed that RPF2 is also in charge of the production of a predominant 5' end for *cox3* mRNA in Col.0 and Ler plants, which is located at position -380 upstream of the translation start codon. The analysis of the *RPF2* gene in Arabidopsis accessions exhibiting *nad9* and *cox3* 5' ends differing from Col.0 revealed that they contained point-mutated or highly rearranged versions of the *RPF2* gene. In these cases, *RPF2*-defective alleles corresponded either to chimeric genic structures between *RPF2* and other *RFL* genes, or *rpf2* copies with numerous non-silent single nucleotide polymorphisms likely preventing RPF2 binding to its RNA targets. Using a similar strategy, the characterization of genetic determinants associated with the differential 5' end processing of *nad4* mRNA in Arabidopsis led to the identification of the *RPF1* gene, encoding another RFL protein. The presence of a functional copy of *RPF1* correlated with the production of a *nad4* mRNA 5' end located 228 nucleotides upstream of the translation start codon, in Col.0 and C24 accessions.[54] *RPF1* alleles in accessions exhibiting *nad4* mRNA 5' end differing from the major one found in Col.0 and C24 plants harbored the same premature stop codon in the *RPF1* coding sequence, preventing the production of a full-length RPF1 protein. Interestingly, *rpf2* and *rpf1* mutants accumulate 5'-extended *nad9*, *cox3*, and *nad4* transcripts, respectively, whose abundances are similar to the 5'-processed mRNAs produced by these proteins. Additionally, these extended transcripts do not affect the accumulation levels of the corresponding mitochondrial proteins, indicating that the 5' extremities generated by RPF1 and RPF2 are not essential for mRNA translation.

In a third study, the analysis of mitochondrial mRNA 5'-ends in a collection of *rfl* T-DNA insertion mutants revealed the function of the *RPF3* gene, and showed that it was essential for the 5' processing of mitochondrial *ccmC* trancripts.[55] Unlike the extended transcripts that accumulate in other *rfl* mutants, the stability of unprocessed *ccmC* transcripts was strongly impaired in *rpf3* plants indicating a possible role of RPF3 in *ccmC* mRNA stabilization. Nevertheless, and despite a severe decrease in CCMC protein accumulation, *rpf3* mutant plants exhibited a wild-type phenotype.

Based on the RPF1, RPF2, and RPF3 assigned functions, one could propose that RFL proteins are factors mostly involved in 5'-end processing of mitochondrial transcripts. Detection of upstream RNA cleavage products strongly suggests that the processing mediated by these proteins involves the recruitment of an endoribonuclease and not a block to exonucleolytic trimming initiated at the 5' extremity of precursor mRNAs, as proposed for other PPR proteins in plastids and mitochondria.[56,57] This mechanism is reminiscent of the mode of action of Rf-PPR proteins, such as RF1A in rice.[6] However, 5'-end processing driven by endonucleolytic cleavage is also associated with mitochondria-targeted PPR proteins that are unrelated to the RFL family, ruling out an exclusive role for RFL proteins in this type of transcript processing.[58,59]

As indicated, the activities supported by the three characterized Arabidopsis RFL do not appear to be essential for the expression of their target mitochondrial transcripts, and corresponding *rfl* mutants do not show any obvious phenotypical alterations compared with wild-type plants. It cannot be ruled out though that the differential processing of mitochondrial mRNA ends could alter unobvious fitness traits, modulating in subtle ways adaptive capacity of the different Arabidopsis accessions harboring distinct alleles of the concerned *RFL* genes. But what appears clearly is that the disruption of these genes does not lead to male sterile plants, and accessions harboring defective *rfl* alleles are fully fertile. Furthermore, genetic targets of characterized RFL proteins correspond to conserved mitochondrial genes. This contrasts with the ones of Rf-PPR in CMS systems, which correspond to transcripts deriving from non-conserved and often recombined mitochondrial ORFs. Nevertheless, restorer loci are often tightly linked to modifier of mitochondrial transcript (*Mmt*) genes, which are associated with internal processing of conserved mitochondrial mRNAs.[60,61] *Mmt* genes may correspond to *RFL* genes located nearby *Rf-PPR* genes or to *Rf-PPR* genes acting on transcripts derived from both normal and CMS-determining mitochondrial genes.[62] In line with this, mutants in a fourth *RFL* gene, namely *AtNG1*, exhibit failures in seed germination, indicating an essential role in mitochondrial functions. However, the RNA target and function of *AtNG1* remain unknown.[63]

The RFL Proteins, Guardians of Proper Mitochondrial Functioning?

As previously developed, the utility of the RNA processing events associated with the characterized Arabidopsis *RFL* genes

appeared to be much less obvious than the functions linked to true restorer genes. Moreover, the expansion of the *RFL* family in autogamous species such as *Arabidopsis thaliana* is surprising and suggests that it is not necessarily associated to the suppression of CMS. Nevertheless, a cross between two phylogenetically distant Arabidopsis accessions recently showed reactivation of a dormant CMS, which could have been inherited from the allogamous ancestors of this species.[64] Although the nuclear determinants controlling this CMS have not been identified yet, this result suggests that ancient CMS may have contributed to the expansion of the *RFL* subfamily in ancestors of autogamous modern species. However, the persistence of *RFL* genes in strictly dioecious species such as *Populus trichocarpa* is rather surprising and strongly supports that *RFL* genes propagation may have been driven by other functional constraints than only the necessity to suppress CMS.[25]

CMS systems have been thought to result from a genomic conflict between nuclear and mitochondrial genomes, which follow different modes of transmission.[2] Indeed, mitochondrial sterilizing factors favor the transmission of maternally inherited cytoplasmic genomes, whereas *Rf* genes re-establish bi-parental transmission of the nuclear genome by suppressing male sterility. This intergenomic conflict has been assimilated to an "arms race," similar to the co-evolution of pathogens effectors and resistance (*R*) genes in the plant-pathogen interactions.[65] The *R* genes are predominantly part of the large family of plant Leu-rich repeat (LRR) proteins, which display a highly dynamic pattern of evolution necessary to adapt to rapidly evolving plant pathogens.[66,67] In several aspects, *RFL* genes are very similar to *LRR* genes: (1) they are often found in clusters of paralogs, (2) unequal crossovers, genomic, and local duplications occur in these clusters and lead to the expansion of the family through a probable "birth-and-death" process, and (3) they undergo a diversifying selection.[24,25] Accordingly, the modular organization of the RFL proteins and the apparent plasticity of their corresponding genes place *RFL* genes as ideal candidates for the surveillance of the mitochondrial genome. It was already suggested that the PPR protein family could have evolved to correct potential deleterious mutations in organelles, but the RFL subfamily could diversify and evolve more rapidly than the rest of the PPR family to actively compensate for mitochondrial genomic deviancies.[18,68] Mitochondrial genomes are known to be highly recombinogenic in plants, resulting in a poor conservation of gene order among related species and in the creation of large number of non-conserved and often chimeric ORF.[69] Both of these resulting phenomenons could necessitate a rapid evolution of correcting factors to prevent the production of long and heterogeneous mitochondrial transcripts or to suppress the action of CMS-inducing ORFs. The RFL genes appear then like a reservoir of fast-evolving genes that has been elected to fulfill these corrective functions. Additionally, several studies have evidenced that the *RFL* family are subjects of tight regulatory controls by small RNA. In *Arabidopsis thaliana* and poplar, several *RFL* genes have been predicted to be or are validated targets of several miRNA and tasiRNA.[70-77] The reasons for this massive targeting by small regulatory RNA is not understood yet, but such a tight regulation could allow the rapid expansion and diversification of the *RFL* subfamily with limiting detrimental effects as previously proposed.[76] All in all, the RFL subfamily is most probably more than a means to cope with male sterility, given the rapid expansion of the family and the apparent tight control by micro-RNAs. Identifying RFL functions in distinct species will certainly help to uncover the functionality of this intriguing subgroup of PPR genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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